AmpliSens® *Enterovirus* 71-FRT PCR kit

RUO

For Professional Use Only

Instruction Manual

KEY TO SYMBOLS USED

REF	Catalogue number	⚠	Caution
LOT	Batch code	Σ	Sufficient for
RUO	Research Use Only		Use-by Date
VER	Version		Consult instructions for use
	Temperature limit		Keep away from sunlight
	Manufacturer	NCA	Negative control of amplification
	Date of manufacture	C–	Negative control of extraction
IC	Internal control	C+ *EV* 71 / STI	Positive control of amplification

1. INTENDED USE

AmpliSens® *Enterovirus* 71-FRT PCR kit is an *in vitro* nucleic acid amplification test for qualitative detection of the RNA of *Enterovirus* type 71 in the biological material (cerebrospinal fluid, fecal samples), taken from the persons suspected of enteroviral infection without distinction of form and presence of manifestation, and natural environments (concentrated water samples) by using real-time hybridization-fluorescence detection of amplified products.

NOTE: For research use only. Not for diagnostic procedures

2. PRINCIPLE OF PCR DETECTION

Enterovirus type 71 detection by the polymerase chain reaction (PCR) is based on the amplification of the pathogen genome specific region using specific *Enterovirus* type 71 primers. In the real-time PCR, the amplified product is detected with the use of fluorescent dyes. These dyes are linked to oligonucleotide probes, which bind specifically to the amplified product during thermocycling. The real-time monitoring of fluorescence intensities during the real-time PCR allows the detection of accumulating product without re-opening the reaction tubes after the PCR run.

AmpliSens® *Enterovirus* 71-FRT PCR kit is a qualitative test that contains the Internal Control (Internal Control STI-87-rec). It must be used in the extraction procedure in order to control the extraction process of each individual sample and to identify possible reaction inhibition.

AmpliSens® *Enterovirus* 71-FRT PCR kit uses "hot-start", which greatly reduces the frequency of nonspecifically primed reactions. "Hot-start" is guaranteed by separation of nucleotides and Taq-polymerase by using a chemically modified polymerase (TaqF). The chemically modified polymerase (TaqF) is activated by heating at 95 °C for 15 min.

The results of amplification are registered in the following fluorescence channels:

Table 1

Channel for fluorophore	FAM	JOE
DNA-target	Internal Control-FL (IC) cDNA	*Enterovirus* 71 cDNA
Target gene	Artificially synthesized sequence	VP3-VP1

3. CONTENT

AmpliSens® *Enterovirus* 71-FRT PCR kit is produced in 1 form:
variant FRT-50 F, REF R-V64-F-CE.

Variant FRT-50 F includes:

Reagent	Description	Volume, ml	Quantity
PCR-mix-FL *EV* 71 / STI	colorless clear liquid	0.6	1 tube
PCR-buffer-C	colorless clear liquid	0.3	1 tube
Polymerase (TaqF)	colorless clear liquid	0.03	1 tube
TM-Revertase (MMlv)	colorless clear liquid	0.015	1 tube
RT-G-mix-2	colorless clear liquid	0.015	1 tube
Positive Control *EV* 71 / STI (C+*EV* 71 / STI)	colorless clear liquid	0.2	1 tube
TE-buffer	colorless clear liquid	0.2	1 tube
Negative Control (C–)*	colorless clear liquid	1.2	1 tube
Internal Control STI-87-rec (IC)**	colorless clear liquid	0.12	5 tubes

* must be used in the extraction procedure as Negative Control of Extraction

** add 10 µl of Internal Control during the RNA extraction procedure directly to the sample/lysis mixture (see **RIBO-sorb** protocol or **RIBO-prep** protocol).

Variant FRT-50 F is intended for 55 reactions (including controls).

4. ADDITIONAL REQUIREMENTS

- RNA extraction kit.
- Disposable powder-free gloves and laboratory coat.
- Pipettes (adjustable).
- Sterile RNase-free pipette tips with aerosol filters (up to 200 µl).
- Tube racks.
- Vortex mixer.
- Desktop centrifuge with a rotor for 2-ml reaction tubes.
- PCR box.
- Real-time instruments (for example, Rotor-Gene 3000/6000 (Corbett Research, Australia); Rotor-Gene Q (QIAGEN, Germany), iCycler iQ5 (Bio-Rad, USA); CFX 96 (Bio-Rad, USA); Mx3000P (Stratagene, USA).
- Disposable polypropylene PCR tubes (0.1- or 0.2-ml):
  a) 0.2-ml PCR tubes with optical transparent domed or flat caps or strips of eight 0.2-ml tubes if a plate-type instrument is used;
  b) 0.2-ml PCR tubes with flat caps or strips of four 0.1-ml Rotor-Gene PCR tubes if a rotor-type instrument is used.
- Refrigerator with the temperature range from 2 to 8 °C.
- Deep-freezer with the temperature range from minus 24 to minus 16 °C.
- Reservoir for used tips.

5. GENERAL PRECAUTIONS

The user should always pay attention to the following:

- Use sterile pipette tips with aerosol filters and use a new tip for every procedure.
- Store all extracted positive material (specimens, controls and amplicons) away from all other reagents and add it to the reaction mix in a distantly separated facility.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Use disposable protective gloves and laboratory cloths, and protect eyes while samples and reagents handling. Thoroughly wash hands afterwards.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- Do not use a kit after its expiration date.
- Dispose of all specimens and unused reagents in accordance with local regulations.
- Samples should be considered potentially infectious and handled in biological cabinet in compliance with appropriate biosafety practices.
- Clean and disinfect all samples or reagents spills using a disinfectant, such as 0.5 % sodium hypochlorite or another suitable disinfectant.
- Avoid inhalation of vapors, samples and reagents contact with the skin, eyes, and mucous membranes. Harmful if swallowed. If these solutions come into contact, rinse the injured area immediately with water and seek medical advice if necessary.
- Safety Data Sheets (SDS) are available on request.
- Use of this product should be limited to personnel trained in DNA amplification techniques.
- Workflow in the laboratory must be one-directional, beginning in the Extraction Area and moving to the Amplification and Detection Area. Do not return samples, equipment and reagents in the area where the previous step was performed.

⚠ Some components of this kit contain sodium azide as a preservative. Do not use metal tubing for reagent transfer.

6. SAMPLING AND HANDLING

NOTE: Obtaining samples of biological materials for PCR-analysis, transportation and storage is described in manufacturer's handbook [1]. It is recommended that this handbook is read before starting work.

AmpliSens® *Enterovirus* 71-FRT PCR kit is intended for analysis of the RNA extracted with RNA extraction kits from different types of biological material (cerebrospinal fluid, fecal samples) and natural environments (concentrated water samples).

The material is to be stored at 2 to 8 °C within 1 day, at the temperature from minus 24 to minus 16 °C within 1 week.

NOTE: Only one freeze-thaw cycle is allowed.

Pretreatment

The pretreatment of cerebrospinal fluid and concentrated water samples is not required.
Fecal samples are to be pretreated.
Samples pretreatment is carried out in accordance with the manufacturer's handbook [1].

7. WORKING CONDITIONS

AmpliSens® *Enterovirus* 71-FRT PCR kit should be used at 18–25 °C.

8. PROTOCOL

8.1. RNA extraction

It is recommended to use the following nucleic acid extraction kits:

- **RIBO-sorb**;
- **RIBO-prep**.

NOTE: Extract RNA according to the manufacturer's protocol.

NOTE: In case of using RIBO-sorb reagent kit, use **10 µl** of **Internal Control STI-87-rec (IC)** per sample.

8.2. Preparing RT-PCR

8.2.1 Preparing tubes for RT-PCR

The total reaction volume is 25 µl, the volume of RNA sample is 10 µl.

1. Prepare the reaction mixture just before use. Prepare the reaction mixture for required number of reactions (including test and control samples) as specified in Table 1

NOTE: Carry out all control amplification reactions (positive and negative) for testing even one test sample. Prepare the reagent mixture for an even number of reactions to attain more precise dispensing.

2. Take the required number of the tubes taking into account the number of test samples and control samples. Select the type of the tubes, stripes and plates according to used device.
3. To prepare the reaction mixture add to a new sterile tube **PCR-mix-FL *EV* 71 / STI**, **PCR-buffer-C**, **RT-G-mix-2, polymerase (TaqF)** and **TM-Revertase (MMlv)** in accordance to Table 2. Thoroughly vortex the tubes and sediment the drops from the caps of the tubes.
4. Transfer **15 µl** of the prepared mixture to each tube

Table 2

Scheme of reaction mixture preparation

		Reagent volume for the specified number of reactions, µl				
Reagent volume per one reaction, µl		**10.00**	**5.00**	**0.25**	**0.50**	**0.25**
Number of test samples	**Number of reactions[1]**	**PCR-mix-FL *EV* 71 / STI**	**PCR-buffer-C**	**RT-G-mix-2**	**Polymerase (TaqF)**	**TM-Revertase (MMlv)**
2	**6**	**60**	**30**	**1.5**	**3.0**	**1.5**
4	**8**	**80**	**40**	**2.0**	**4.0**	**2.0**
6	**10**	**100**	**50**	**2.5**	**5.0**	**2.5**
8	**12**	**120**	**60**	**3.0**	**6.0**	**3.0**
10	**14**	**140**	**70**	**3.5**	**7.0**	**3.5**
12	**16**	**160**	**80**	**4.0**	**8.0**	**4.0**
14	**18**	**180**	**90**	**4.5**	**9.0**	**4.5**
16	**20**	**200**	**100**	**5.0**	**10.0**	**5.0**
18	**22**	**220**	**110**	**5.5**	**11.0**	**5.5**
20	**24**	**240**	**120**	**6.0**	**12.0**	**6.0**
22	**26**	**260**	**130**	**6.5**	**13.0**	**6.5**
24	**28**	**280**	**140**	**7.0**	**14.0**	**7.0**
26	**30**	**300**	**150**	**7.5**	**15.0**	**7.5**
28	**32**	**320**	**160**	**8.0**	**16.0**	**8.0**

5. Add **10 µl** of **RNA samples** extracted from test or control samples of RNA extraction stage using tips with filter. Discard the unused reaction mixture.

NOTE: Avoid transferring of sorbent together with the RNA samples extracted by RIBO-sorb kit.

6. Carry out the control amplification reactions:

NCA – Add **10 µl** of **TE-buffer** to the tube labeled NCA (Negative Control of Amplification).

C+$_{EV71\ /\ STI}$ – Add **10 µl** of **Positive Control *EV* 71 / STI (C+$_{EV\ 71\ /\ STI}$)** to the tube labeled **C+$_{EV\ 71\ /\ STI}$** (Positive Control of Amplification).

C– – Add **10 µl** of **the sample extracted from the Negative Control reagent** to the tube labeled C–.

8.2.2. Reverse transcription and amplification

NOTE: Make sure that the amplification run starts within 10-15 min after the addition of RNA to the reaction mixture

1. Create a temperature profile on your instrument as follows:

Table 3

AmpliSens unified amplification program for rotor-type[2] and plate-type instruments[3]

Step	Temperature, °C	Time	Fluorescence detection	Cycles
1	**50**	**15 min**	**–**	**1**
2	**95**	**15 min**	**–**	**1**
3	**95**	**10 s**	**–**	**45**
	60	**20 s**	**FAM, JOE**	

NOTE: Any combination of the tests can be performed in one instrument simultaneously with the use of the unified amplification program

Note – When several tests are performed simultaneously the detection in all used channels is enabled.

2. Adjust the fluorescence channel sensitivity according to *Important Product Information Bulletin* and Guidelines [2].
3. Insert tubes into the reaction module of the device.

NOTE: It is recommended to sediment drops from walls of tubes by short centrifugation (1–3 s) before placing them into the instrument.

4. Run the amplification program with fluorescence detection.
5. Analyze results after the amplification program is completed.

9. DATA ANALYSIS

Analysis of results is performed by the software of the real-time PCR instrument used by measuring fluorescence signal accumulation in two channels:

– The signal of the IC cDNA amplification product is detected in the channel for the FAM fluorophore.
– The signal of the *Enterovirus* type 71 cDNA amplification product is detected in the channel for the JOE fluorophore.

Results are interpreted by the crossing (or not-crossing) the fluorescence curve with the threshold line set at a specific level that corresponds to the presence (or absence) of a *Ct* value of a cDNA sample in the corresponding column of the result grid.
The results are interpreted in accordance with the Table 4 and *Important Product Information Bulletin*:

Table 4

Results interpretation

***Ct* value in the channel for fluorophore**		**Result**
FAM	**JOE**	
< boundary value	absent or > boundary value	*Enterovirus* type 71 RNA **is not detected**
> or < boundary value	< boundary value	*Enterovirus* type 71 RNA **is detected**
absent or > boundary value	absent or > boundary value	**Invalid** result Repeat the PCR-analysis from the extraction stage

NOTE: Boundary *Ct* values are specified in the *Important Product Information Bulletin* enclosed in the PCR kit. See also Guidelines [2].

The result of the analysis is considered reliable only if the results obtained for Positive and Negative Controls of amplification as well as for the Negative Control of extraction are correct (see Table 5).

Table 5

Results for controls

Control	**Stage for control**	***Ct* value in the channel for fluorophore**	
		FAM	**JOE**
C–	RNA extraction	< boundary value	absent or > boundary value
NCA	RT-PCR	absent or > boundary value	absent or > boundary value
C+	RT-PCR	< boundary value	< boundary value

[1] Number of test samples (N) + 1 control of RNA extraction + 2 controls of RT-PCR + 1 extra reaction (N+1+2+1).

[2] For example, Rotor-Gene 3000, Rotor-Gene 6000 (Corbett Research, Australia), Rotor-Gene Q (QIAGEN, Germany).
[3] For example, iCycler iQ5 (Bio-Rad, USA), Mx3000P (Stratagene USA).

10. TROUBLESHOOTING

The results of the analysis are not taken into account in the following cases:

1. If the *Ct* value determined for the Positive Control of Amplification (C+) in the channel for JOE fluorophore is greater than the boundary *Ct* value or absent, the RT-PCR and detection should be repeated for all samples in which the *Enterovirus* type 71 RNA was not detected.
2. If the *Ct* value determined for the Negative Control of Amplification (NCA) and/or Negative Control of Extraction (C–) in the channel for JOE fluorophore is less than the boundary *Ct* value, PCR analysis (beginning with RNA extraction stage) should be repeated for all samples in which the *Enterovirus* type 71 RNA was detected.

11. TRANSPORTATION

AmpliSens® *Enterovirus* 71-FRT PCR kit should be transported at 2–8 °C for no longer than 5 days.

12. STABILITY AND STORAGE

All components of the **AmpliSens® *Enterovirus* 71-FRT** PCR kit are to be stored at 2–8 °C when not in use (except for PCR-mix-FL *EV* 71 / STI, PCR-buffer-C, RT-G-mix-2, Polymerase (TaqF), TM-Revertase (MMlv)). All components of the **AmpliSens® *Enterovirus* 71-FRT** PCR kit are stable until the expiry date stated on the label. The shelf life of reagents before and after the first use is the same, unless otherwise stated.

NOTE: PCR-mix-FL *EV* 71 / STI, PCR-buffer-C, RT-G-mix-2, Polymerase (TaqF), TM-Revertase (MMlv) are to be stored at the temperature from minus 24 to minus 16 °C.

NOTE: PCR-mix-FL *EV* 71 / STI is to be kept away from light.

13. SPECIFICATIONS

13.1. Analytical sensitivity

Biological material	Pathogen agent	Nucleic acid extraction kit	PCR kit	Sensitivity, GE/ml[4]
Cerebrospinal fluid, concentrated water samples	*Enterovirus* type 71	RIBO-sorb RIBO-prep	variant FRT-50 F	5×10^3
Fecal samples	*Enterovirus* type 71	RIBO-sorb RIBO-prep	variant FRT-50 F	1×10^4

13.2. Specificity

The analytical specificity of **AmpliSens® *Enterovirus* 71-FRT** PCR kit is ensured by selection of specific primers and probes as well as stringent reaction conditions. The primers and probes were checked for possible homologies to all sequences published in gene banks by sequence comparison analysis.

The specificity was proved on the follows strains of microorganisms: *Human enterovirus* (representatives of different genetic clusters – *Human echovirus* 2, 6, 9, 11, 14, 15, 16, 17, 18, 30; *Human coxsackievirus* A4, A5, A6, A9, A16, B4, B5, *Human poliovirus* 1, 2, 3 (Sabin1, Sabin2, Sabin3)); *Influenza viruses* A (H13N2, H9N2, H8N4, H2N3, H4N6, H11N6, H12N5, H3N8, H1N1, H6N2, H10N7, H5N1), B, *Rhinovirus*, *RS* viruses, *Human adenovirus* types 3, 5, 7, 37, 40, 41 (clinical isolates, the specificity was proved by direct sequencing of nucleic sequences); microorganisms strains and DNA samples – human DNA, strains of *Acinetobacter baumanii* ATCC® 19606™, *Bacteroides fragilis* ATCC® 25285™, *Bordetella bronchiseptica* ATCC® 10580™, *Bordetella bronchiseptica* ATCC® 4617™, *Bordetella pertussis* ATCC® 9340™, *Candida albicans* ATCC® 14053™, *Candida guilliermondii* ATCC® 6260™, *Candida krusei* ATCC® 14243™, *Clostridium difficile* ATCC® 9689™, *Clostridium septicum* ATCC® 12464™, *Corynebacterium jeikeium* ATCC® 43734™, *Corynebacterium xerosis* ATCC® 373™, *Eggerthella lenta (Eubacterium lentum)* ATCC® 43055™, *Enterobacter aerogenes* ATCC® 13048™, *Enterobacter cloacae* ATCC® 13047™, *Enterococcus faecalis* ATCC® 29212™, *Enterococcus faecalis (vancomycin resistant)* ATCC® 51299™, *Enterococcus faecium* ATCC® 35667™, *Erysipelothrix rhusiopathiae* ATCC® 19414™, *Escherichia coli* ATCC® 25922™, *Escherichia coli* ATCC® 35218™, *Fluoribacter (Legionella) dumoffii* ATCC® 33279™, *Haemophilus infuenzae* ATCC® 33930™, *Haemophilus influenzae* ATCC® 9006™, *Haemophilus influenzae* ATCC® 10211™, *Haemophilus parainfluenzae* ATCC® 7901™, *Klebsiella oxytoca* ATCC® 49131™, *Klebsiella pneumoniae* ATCC® 27736™, *Legionella pneumophila* ATCC® 33152™, *Listeria grayi (murrayi)* ATCC® 25401™, *Listeria innocua* ATCC® 33090™, *Listeria monocytogenes* ATCC® 7644™, *Moraxella (Branhamella) catarrhalis* ATCC® 25238™, *Moraxella (Branhamella) catarrhalis* ATCC® 8176™, *Neisseria meningitidis* ATCC® 13102™, *Neisseria meningitidis* ATCC® 13090™, *Neisseria lactamica* ATCC® 23970™, *Neisseria gonorrhoeae* ATCC® 19424™, *Neisseria gonorrhoeae* ATCC® 49926™, *Peptoniphilus (Peptostreptococcus) anaerobius* ATCC® 27337™, *Proteus mirabilis* ATCC® 12453™, *Proteus vulgaris* ATCC® 6380™, *Propionibacterium acnes* ATCC® 11827™, *Pseudomonas aeruginosa* ATCC® 15442™, *Rhodococcus equi* ATCC® 6939™, *Salmonella enterica subsp. enterica serovar Typhimurium* ATCC® 14028™, *Serratia marcescens* ATCC® 14756™, *Staphylococcus aureus* ATCC® 6538P™, *Staphylococcus aureus* (MRSA) ATCC® 43300™, *Staphylococcus aureus* ATCC® 29213™, *Staphylococcus aureus* ATCC® 25923™, *Staphylococcus aureus* ATCC® 33862™, *Staphylococcus aureus* (MRSA) ATCC® 33591™, *Staphylococcus aureus subsp. aureus* ATCC® 12600™, *Staphylococcus epidermidis* ATCC® 12228™, *Staphylococcus haemolyticus* ATCC® 29970™, *Staphylococcus saprophyticus* ATCC® 49907™, *Stenotrophomonas maltophilia* ATCC® 13637™, *Streptococcus agalactiae* ATCC® 12386™, *Streptococcus agalactiae* ATCC® 13813™, *Streptococcus equisimilis* ATCC® 12388™, *Streptococcus equi subsp. equi* ATCC® 9528™, *Streptococcus bovis* (Group D) ATCC® 9809™, *Streptococcus mutans* ATCC® 35668™, *Streptococcus pneumoniae* ATCC® 49619™, *Streptococcus pneumoniae* ATCC® 6303™, *Streptococcus pneumoniae* ATCC® 27336™, *Streptococcus pneumoniae* ATCC® 6305™, *Streptococcus pyogenes* ATCC® 19615™, *Streptococcus salivarius* ATCC® 13419™, *Streptococcus uberis* ATCC® 700407™, *Trichophyton mentagrophytes* ATCC® 9533™, *Vibrio parahaemolyticus* ATCC® 17802™, *Vibrio vulnificus* ATCC® 27562™, *Moraxella catarrhalis* ATCC® 25240™. Nonspecific responses were absent in tests of DNA samples of this microorganisms and human DNA samples.

13.3. Reproducibility and repeatability

Biological material	Number of repeats	Coefficient of variation CV, %
Dispersion of values in a single test		
Fecal samples	8	0.49
Concentrated water samples	8	0.49
Cerebrospinal fluid	8	1.21
Dispersion of values between tests, carried out in different days		
Fecal samples	16	1.72
Concentrated water samples	16	2.27
Cerebrospinal fluid	16	1.91

14. REFERENCES

1. Handbook “Sampling, Transportation, and Storage of Clinical Material for PCR diagnostics”, developed by Federal Budget Institute of Science “Central Research Institute for Epidemiology” of Federal Service for Surveillance on Consumers’ Rights Protection and Human Well-Being.
2. Guidelines to the **AmpliSens® *Enterovirus* 71-FRT** PCR kit for qualitative detection of RNA of *Enterovirus* type 71 in the biological material (cerebrospinal fluid, fecal samples) and natural environments (concentrated water samples) by real-time hybridization-fluorescence detection of amplified products developed by Federal Budget Institute of Science “Central Research Institute for Epidemiology”.

15. QUALITY CONTROL

In compliance with the Federal Budget Institute of Science “Central Research Institute for Epidemiology” ISO 13485-Certified Quality Management System, each lot of **AmpliSens® *Enterovirus* 71-FRT** PCR kit has been tested against predetermined specifications to ensure consistent product quality.

AmpliSens®

Federal Budget Institute of Science “Central Research Institute for Epidemiology”
3A Novogireevskaya Street
Moscow 111123 Russia

[4] Genome equivalents (GE) of the pathogen agent per 1 ml of a sample.